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# Age Determination by Skeletochronology of *Rana nigrovittata*, a Frog from Tropical Forest of Thailand

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**ABSTRACT**— Age in 96 individuals of a tropical frog *Rana nigrovittata* from Thailand was skeletochronologically investigated. Hematoxylinophilic lines interpreted as lines of arrested growth (LAGs) were observed clearly in the periosteal bone suggesting that this technique can be used to estimate the age of frogs from tropical Asia. The maximum age observed was nine years in males and six in females, but age structure did not differ significantly between the sexes. In the same age class, males were significantly larger than females.

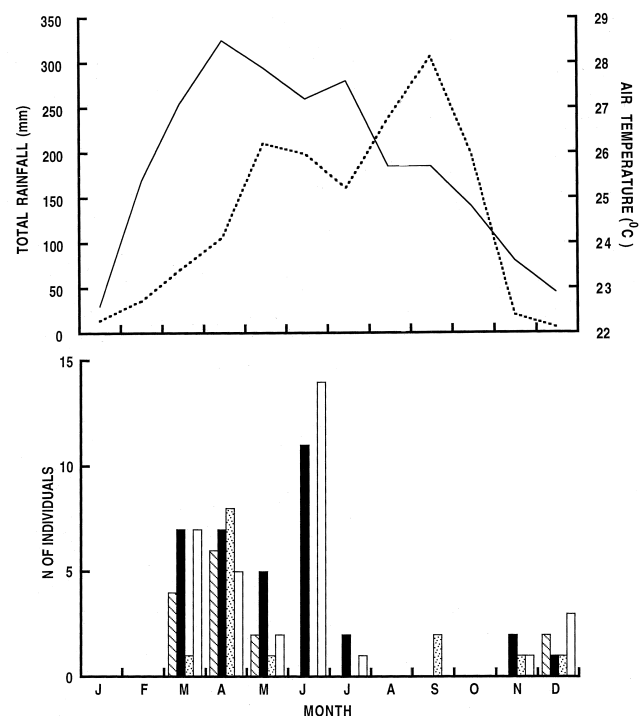
## INTRODUCTION

Many recent studies have documented that skeletochronological method is applicable to determine the individual age of amphibians in nature (e.g. Hemelaar, 1988; Kusano *et al.*, 1995a; Sagor *et al.*, 1998). The method is based on the presence of growth layers recorded in cross-sections of long bones (Halliday and Verrell, 1988; Castanet and Smirina, 1990). This technique has been proved to be an excellent tool for investigating population age structure without sacrificing specimens (e.g., Francillon-Vieillot *et al.*, 1990; Roger and Harvey, 1994; Kusano *et al.*, 1995a). However, as pointed out by Halliday and Verrell (1988), there are very few studies for amphibians from tropical regions. Most of previous skeletochronological studies have ever been conducted in species from temperate regions, such as, *Bufo bufo* (Hemelaar, 1988), *Bufo calamita* (Denton and Beebee, 1993), *Rana sylvatica* (Bastein and Leclair, Jr., 1992), *Rana tagoi* (Kusano *et al.*, 1995a), and *Rana sakuraii* (Kusano *et al.*, 1995b). Much smaller numbers of studies have been made for desert species such as *Bufo pentoni* (Barbault *et al.*, 1979) and *Scaphiopus couchii* (Tinsley and Tocque, 1995), and only a single study dealt with a mantellid, *Mantidactylus microtypanum* from the tropical rain forest of Madagascar (Guarino *et al.*, 1998). Thus no studies have ever been made for amphibians from Asian tropics. The present study was made to examine the applicability of skeletochronological method for amphibians from tropical Asia, using a frog *Rana nigrovittata* from Thailand. We also studied the age structure, longevity, and growth pattern of this species on the basis of individual ages estimated.

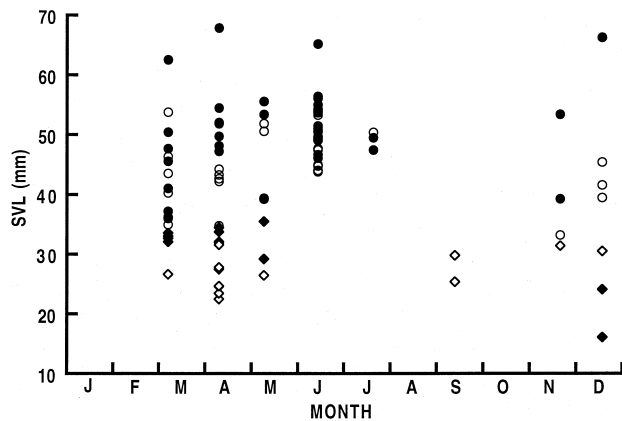
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## MATERIALS AND METHODS

*Rana nigrovittata* is widely distributed from Assam to Southern China, Vietnam, and south to Peninsular Malaysia (Frost, 1985), and inhabits on small pools, banks of rivers, small streams, and edges of lakes (Taylor, 1962).



**Fig. 1.** The relationship between the date of collection and the number of individuals collected (below) and values of the average monthly total rainfall (dotted line) and average temperature (solid line) of Chachoengsao Wildlife Research Center (top). Closed squares, hatched squares, open squares, and dotted squares indicate adult males, juvenile males, adult females, and juvenile females, respectively.



**Fig. 2.** The relationship between the date of collection and frog SVL. Closed circles, closed diamonds, open circles, and open diamonds indicate adult males, juvenile males, adult females, and juveniles females, respectively.

Sampling was made in the Chachoengsao Wildlife Research Center, eastern Thailand (13°24'17"N, 101°52'17"E, alt. 30–150 m). The study area is in a mainly dry evergreen forest with small patchy area of moist evergreen, moist-mixed species forest and dipterocarp dominated forest. Monthly climatic data of the area are shown in Fig. 1. The dry season in this area occurs from November to February with a total rainfall of 0–60 mm per month.

A small stream in a dry evergreen forest was chosen for studying of *R. nigrovittata*. Surveys were carried out from March to July, September, and November to December of 1996, and a total number of 96 frogs were captured. Frogs were seen in and along the stream during the dry season while they were found on the bank in the rainy season. Frogs collected were anesthetized by chloroform and preserved in 70% alcohol. The snout to vent length (SVL) was measured to the nearest 0.5 mm with a vernier caliper. We checked sex of each specimen by dissection. Sexual maturity was determined by the pres-

ence of secondary sexual characters such as humeral gland and nuptial pad in males while female maturity was determined by the presence of large pigmented ova and convoluted oviducts. As a result, we examined 28 juveniles (14 males and 14 females) and 68 adults (35 males and 33 females).

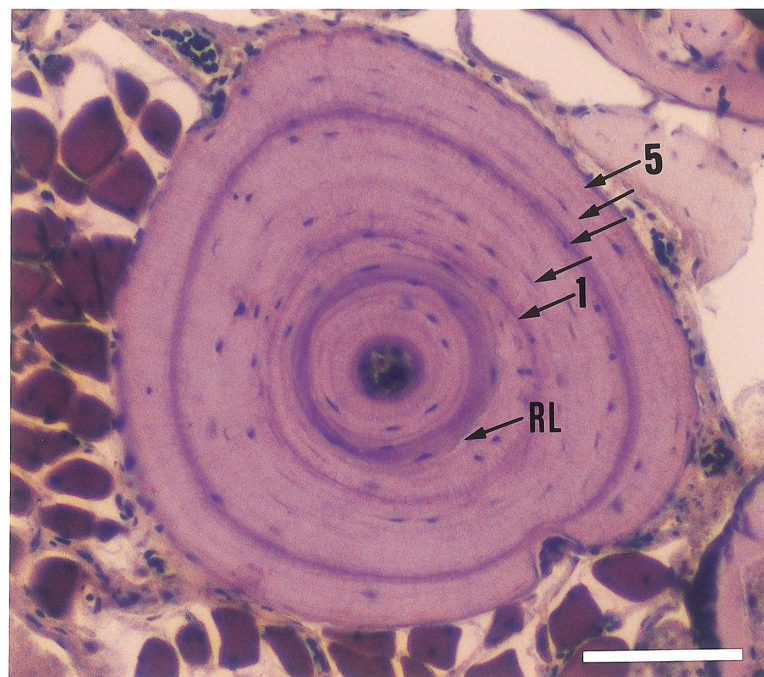
In the study area, the breeding season of *R. nigrovittata* occurs from April to June when the frequency of occurrence of adult frogs was higher (Figs. 1–2) and majority of females contained mature eggs in their ovaries. Although the larval period and time of metamorphosis at the study site is unclear, the smallest frog with an SVL of 16.1 mm was captured in December.

The third phalanges of the third digit of the left hand taken from preserved specimens were washed in running water for 24 hr, decalcified in 5% nitric acid for 60–90 min, and washed in the running water for 24 hr. It was cross-sectioned (20–22  $\mu$ m thick) by a freezing microtome and stained with hematoxylin (Mayer's acid hemalum) for 30 min. Since resting line was absent or incomplete in the epiphysis, sections from the central region of diaphysis were selected, and mounted in glycerin after rinsed in the tap water. Sections were examined under a light microscope and the number of lines of arrested growth (LAGs) presented in the periosteal bone was counted. To specify the age of each individual frog, the time of capture in the year was taken into consideration. Diameters of the innermost LAG and of the innermost portions of the periosteal bone were measured using an ocular micrometer.

We examined sexual difference in age composition by Mann-Whitney U-test. For body size difference between sexes, Student's t-test was applied. Logistic Model  $L = a / (1 + e^{-bt})$ , where L is the SVL (mm) at time t (yr), a is the asymptotic size; c is the constant determined by putting 0 to t; and b is the instantaneous growth rate, was used to estimate the growth pattern (Misawa and Matsui, 1999). The significance level was set at 0.05.

## RESULTS

The cross-sections of phalangeal bones were nearly circular in shape, and hematoxylinophilic lines interpreted as



**Fig. 3.** Five LAGs seen in a phalangeal bone of an adult female *R. nigrovittata* with the body size in SVL of 51.9 mm and collected on 8 May 1996. As seen in the figure, the 2<sup>nd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> LAGs are not clear compared with 1<sup>st</sup> and 3<sup>rd</sup> ones. RL=resorption line. Scale bar=100  $\mu$ m.

**Table 1.** Average diameters (in  $\mu$ ) of the innermost portion of periosteal bone and the innermost LAG.

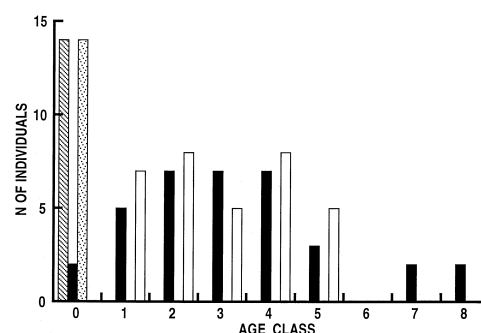
Age class	N of LAG	Innermost portion of periosteal bone		Innermost LAG	
		N	Average $\pm$ 2SE	N	Average $\pm$ 2SE
0	0	6	105 $\pm$ 26	0	—
1	1	24	101 $\pm$ 12	24	170 $\pm$ 10
	2	10	133 $\pm$ 36	10	209 $\pm$ 50
2	2	6	162 $\pm$ 24	6	183 $\pm$ 28
	3	9	157 $\pm$ 14	9	196 $\pm$ 16
3	3	11	156 $\pm$ 20	11	203 $\pm$ 14
	4	1	130	1	170
4	4	9	143 $\pm$ 18	9	199 $\pm$ 20
	5	6	175 $\pm$ 14	6	215 $\pm$ 26
5	5	8	170 $\pm$ 16	8	201 $\pm$ 18
6	—	—	—	—	—
7	6	2	255	2	285
8	7	2	235	2	265

LAGs were observed in the periosteal tissue, which was separated from the endosteal tissue by a resorption line (RL in Fig. 3). Although LAGs were not uniformly clear (Fig. 3), we could count their number by closely examining several sections for each individual. Age was calculated on the basis of the number of LAG and the time of the year when the frog was captured. In juvenile frogs captured from September to December, the LAG was not found in the phalangeal bones. By contrast, one LAG was found closer to the outer margin of the periosteal bone tissue in juveniles collected from March to May, suggesting these juveniles being collected shortly after formation of the LAG. These results indicate that the first LAG is formed during the dry season between November and February. Thus, when the starting point is set at June (at the end of the breeding season), frogs in the age class 0 included both without LAG (from June to the next February) and with one LAG (from March to May of the next year). Similarly, each age class included frogs both with LAGs corresponding to that age and with one additional LAGs (Table 1).

Because the periosteal tissue including the inner LAGs may be resorbed through the growth of the endosteal tissue, we measured diameters of the innermost portion of the periosteal bone (=resorption line) and of the innermost LAG to

detect the resorption process. The result indicated that some part of the periosteal bone tissue was actually resorbed and that this resorption process was enhanced with the increase of the age. Four individuals with 6 LAGs (2 individuals) and 7 LAGs (2 individuals) had apparently their first LAG resorbed, and one year was added to estimate their ages (Table 1).

Population age structure differed insignificantly between the sexes (Mann-Whitney U-test;  $P > 0.05$ , Fig. 4). Age of adult

**Fig. 4.** Population age structure for male ( $n=49$ ) and female ( $n=47$ ) *Rana nigrovittata*. Closed squares, hatched squares, open squares, and dotted squares indicate adult males, juvenile males, adult females, and juveniles females, respectively.**Table 2.** Number of individuals, mean SVL in mm, and range, for each age class.

Age class	Male			Female		
	N	Average $\pm$ 2SE	Range	N	Average $\pm$ 2SE	Range
0	16	30.3 $\pm$ 2.9	16.1–37.2	14	28.0 $\pm$ 1.7	22.5–32.0
1	5	40.9 $\pm$ 2.4	39.3–45.6	7	36.9 $\pm$ 2.6	33.2–42.7
2*	7	48.4 $\pm$ 1.4	46.7–51.9	8	43.6 $\pm$ 1.8	39.5–47.5
3*	7	49.6 $\pm$ 1.3	46.1–51.5	5	44.5 $\pm$ 1.1	43.3–46.4
4*	7	54.3 $\pm$ 1.1	52.1–56.4	8	50.2 $\pm$ 0.9	47.7–51.9
5*	3	54.9 $\pm$ 1.3	54.0–56.1	5	53.0 $\pm$ 1.3	50.5–53.9
6	—	—	—	—	—	—
7	2	64.5	62.6–66.3	—	—	—
8	2	66.6	65.2–67.9	—	—	—

\* = Significant difference between the sexes by student's t-test,  $P < 0.05$

frogs differed insignificantly between the sexes (mean $\pm$ 2SE =3.70 $\pm$ 0.68 yrs in 35 males and 3.29 $\pm$ 0.42 yrs in 33 females; Mann-Whitney U-test,  $P>0.05$ ). The maximum age that was observed in *R. nigrovittata* in this study was 8.8 years in males and 5.7 year in females. Thus, the maximum life span for *R. nigrovittata* is estimated to be at least 8.8 years.

The smallest adult male was 36.3 mm (in SVL) with 0.7 yr of age and the smallest females was 33.2 mm with 1.4 yr. The largest size was 67.9 mm in a 8.8 yr male and 53.9 mm in a 5.0 yr female. Adult males were significantly larger than females (mean $\pm$ 2SE: 50.6 $\pm$ 2.6 mm in 35 males and 45.3 $\pm$ 2.1 mm in 33 females: t-test,  $P<0.05$ ), and this was the case in each age group (Table 2).

Individuals of different ages greatly overlapped in body size as seen in Table 2 and the body size proved to be not good indicator of age. Thus, growth curves estimated by a Logistic Model (Fig. 5) indicated that sexes did not differ in the instantaneous growth rate ( $b\pm 2SE=0.5805\pm 0.1494$  in males and  $0.5587\pm 0.1527$  in females) or in the asymptotic size ( $a\pm 2SE=62.72\pm 4.32$  in males and  $57.35\pm 5.23$  in females), probably due to great variation in SVL.

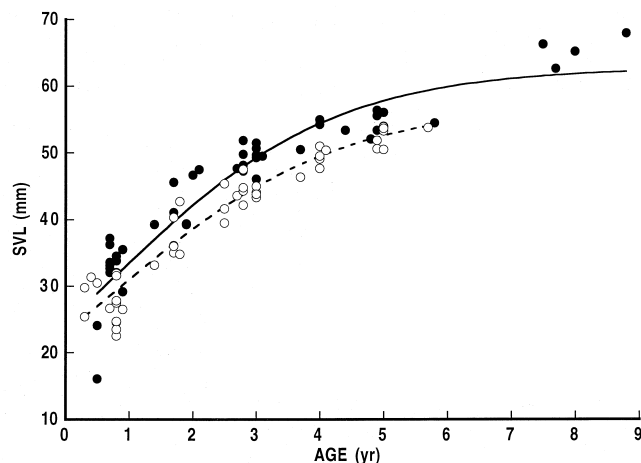


Fig. 5. Growth curves for *Rana nigrovittata* as determined by a logistic model. The closed and open circles indicate males  $L=62.7/(1+e^{0.45-0.58t})$  and females  $L=57.4/(1+e^{0.40-0.56t})$ , respectively.

## DISCUSSION

From the results of the present study, the technique of skeletochronology proved to be successfully applied for Asian tropical frog at least for *R. nigrovittata*. In the case of *Mantidactylus microtypanum*, a single tropical species by now examined, LAGs are reported to be formed by the harsh annual period, with decreasing temperature, rainfall, and available food (Guarino *et al.*, 1998). On the other hand, LAGs of *Bufo pentoni* living in the subdesert are considered to be formed by a long aestivation (Barbault *et al.*, 1979), and LAGs *Geocrinia alba* and *G. vitellina* are similarly estimated to be formed during the dry months (Driscoll, 1999). In our study area, the dry season occurred from November to February and the number of frogs found was not so large in this period

(Fig. 1). In addition, LAG of juvenile frog was closer to the outer edge of phalangeal bone cross-section. It is, therefore, highly probable that LAG is formed in the dry season (November to February) in *R. nigrovittata*. The temperature is also low in this season (Fig. 1) and probably activities of frogs would be lower resulting in the formation of resting lines.

Forester and Lyken (1991) reported that periosteal bone is eroded over a time in *Notophthalmus viridescens*. Verrell and Francillon (1986) similarly noted that endosteal bone may be deposited after the innermost LAG has been eroded in *Triturus vulgaris*. We also observed the endosteal bone and the occurrence of resorption in the innermost portion of the periosteal tissue in most phalangeal bones. Thus the simple counting of LAGs may underestimate true age especially in older individuals and diameters of the resorption line and inner LAG should be measured and compared for accurate estimation of individual ages.

Compared with a frog from a temperate region (e.g. *R. rugosa* and *R. nigromaculata*: Khonsue, unpublished data) the distinctness or intensity of LAGs was less conspicuous in *R. nigrovittata*. This finding supports an assumption made by Castanet and Smirina (1990) that the optical sharpness sometimes decreases and LAGs can be more or less inconspicuous in bones of specimens living under a more constant climatic condition.

Our finding confirmed that body size is a poor indicator for estimating the age of frogs. This tendency has been pointed out by many previous authors (Halliday and Verrell, 1988; Guarino *et al.*, 1998; Tinsley and Tocque, 1995).

Shine (1979) reported females were larger than males in more than 90% of 589 anuran species he studied. Male *R. nigrovittata* contrariwise proved to be significantly larger than females. Larger body size in male frogs has been discussed in relation to male-male combating behavior (Shine, 1979). Our field observation, however, never proves presence of such behavior in *R. nigrovittata*, and further studies are necessary to explain reversed sexual dimorphism in this species.

The longevity of *R. nigrovittata* was estimated to be at least nine yrs in males and six yrs in females. This is the first indication of longevity in *R. nigrovittata* in nature. Longevity observed in the other tropical species *M. microtypanum* was seven yrs (Guarino *et al.*, 1998). Longevities of these two tropical species seem to be shorter than those of some temperate species such as *Bufo bufo* (12 yrs: Hemelaar, 1988) and *Bombina variegata* (11–14 yrs: Plytycz and Bigaj, 1993). Hemelaar (1988) demonstrated that populations of *Bufo bufo* from higher altitudes and more northern latitudes were older than those from lower altitudes and more southern latitudes. Different longevity of frogs is regarded as being constrained by the mortality rate, and it is generally accepted that a high mortality of animals occurs due to a large number of predators in the tropical region. Ballinger (1979) pointed out that predation was the major factor of mortality in a lizard species at lower altitudes. On the other hand, Ryser (1996) attributed shorter period of activity to reduction of risks to be killed in the temperate regions. We need future detailed field studies of

predation and activity pattern in *R. nigrovittata*.

In conclusion, the present result demonstrates that skeletochronological technique using phalangeal bone can be used to estimate the age of a tropical species *R. nigrovittata*. This species occurs mainly in the Savanna where annual climate condition is less constant than in tropical rain forest. Therefore, further studies using many other tropical Asian frog species, especially those from tropical rain forests, are necessary to confirm the generality of the present results.

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